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Regulation of Ubiquitin and Ubiquitin-like Modifiers by Phosphorylation

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Summary

The regulatory influence of ubiquitin is vast, encompassing all cellular processes, by virtue of its central roles in protein degradation, membrane trafficking, and cell signaling. *But how does ubiquitin, a 76 amino acid peptide, carry out such diverse, complex functions in eukaryotic cells?* Part of the answer is rooted in the high degree of complexity associated with ubiquitin polymers, which can be “read” and processed differently depending on topology and cellular context. However, recent evidence indicates that post-translational modifications on ubiquitin itself enhance the complexity of the ubiquitin code. Here, we review recent discoveries related to the regulation of the ubiquitin code by phosphorylation. We summarize what is currently known about phosphorylation of ubiquitin at Ser65, Ser57 and Thr12, and we discuss the potential for phosphorylation of ubiquitin at other sites. We also discuss accumulating evidence that ubiquitin-like modifiers, such as SUMO, are likewise regulated by phosphorylation. A complete understanding of these regulatory codes and their complex lexicon will require dissection of mechanisms that govern phosphorylation of ubiquitin and ubiquitin-like proteins, particularly in the context of cellular stress and disease.

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Author Contributions

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Conflicts of Interest

Eicke Latz is co-founder and consultant of IFM Therapeutics, DiosCure Therapeutics and Odyssey Therapeutics.

For all other authors there are no conflicts of interest to declare.

Keywords

ubiquitin; phosphorylation; signalling; stress responses; proteostasis; mitophagy; protein degradation

Introduction

Post-translational modification of proteins by conjugation to ubiquitin (Ub) is critical for cellular adaptations that involve remodeling the proteome in response to a changing environment or exposure to stress. Decades of intense research have been dedicated to interrogating ubiquitin, and yet we still do not fully understand how this 76 amino acid peptide exhibits such broad utility as a regulatory modifier. Part of the answer is rooted in the high degree of complexity associated with Ub polymers, which can be “read” and processed differently depending on topology and cellular context. This is referred to as the *ubiquitin code*. One layer of this code is based on the formation of linkage-specific polymers by the Ub conjugation enzymes (E1-E2-E3 cascades) [1–3] combined with regulated disassembly of Ub polymers by deubiquitylases (DUBs) [4]. However, recent evidence indicates the code is further enhanced by the formation of mixed and branched polymers [5, 6] and by post-translational modifications (PTMs) on Ub itself [7, 8]. These recent findings reveal that the ubiquitin code is more complex than previously appreciated. A complete understanding of its lexicon will require a deeper interrogation of how complex topologies and ubiquitin PTMs contribute to modified substrates’ regulatory fate.

One emerging paradigm for lexicon expansion of the ubiquitin code involves phosphorylation of ubiquitin itself (Figure 1). The first report describing the detection of phosphorylated ubiquitin was published in 2003 [9] and subsequently numerous studies have reported proteomic evidence of Ub phosphorylation at multiple sites. However, understanding the biological functions of ubiquitin phosphorylation has been slow, likely due to the technical challenges associated with detecting phosphorylated ubiquitin species. However, enthusiasm for the biology of ubiquitin phosphorylation was bolstered in 2014 when a series of studies reported that Ser65-phosphorylated ubiquitin regulates PINK1-Parkin-mediated mitophagy [10–13], providing the first evidence of functional regulation of ubiquitin by phosphorylation. Since then, additional roles for phosphorylated ubiquitin have been reported (Table 1), and there is emerging evidence that phosphorylation of ubiquitin-like modifiers (UBLs) also confers functional regulation. In this review, we summarize recent findings related to the regulation of Ub and UBLs by phosphorylation, discuss critical knowledge gaps that remain, and propose experimental directions and strategies for catalyzing discoveries that will enhance our understanding of ubiquitin phosphorylation and the regulatory lexicon of the ubiquitin code.

Ser65 Phosphorylation of Ubiquitin

Ser65 phosphorylation (pS65) is the most extensively studied phosphorylation event on Ub based on its role in mitochondrial homeostasis regulation [10, 14, 15]. When mitochondria become damaged in healthy cells, mitochondrial proteins in the damaged region are conjugated to pS65-Ub, which targets destruction by autophagosomal degradation

(mitophagy) in a tightly regulated signaling cascade. AQUA-based quantification data revealed that pS65-Ub is <0.1% of the total Ub in unperturbed human cells but the level increases to 2% in cells treated with mitochondria-damaging agents. Global mitochondrial depolarization enriches pS65 ubiquitylation of proteins at the outer mitochondrial membrane (OMM), reaching roughly 20% of the total mitochondrial Ub [13]. In yeast, pS65-Ub comprises <0.5% of total Ub at a steady-state, approximately as abundant as pSer57 [16]. Outside its role in maintaining mitochondrial health, the biology of pS65-Ub in a cellular context is poorly understood. Although Ser65 is non-essential as demonstrated by alanine substitution [17], it is part of the β 5-bulge adjacent to Lys63 [18] (Figure 2), a site for K63-linked Ub polymerization that modulates DNA repair [19], cell-cycle control [20], transcriptional regulation [21], endocytic trafficking [22], and inflammatory signaling [23].

PINK1 (PTEN induced putative kinase 1) is the only kinase known to phosphorylate the Ser65 residue of free and conjugated Ub, which has been demonstrated *in vitro* and *in vivo* [10, 11, 24]. In the absence of mitochondrial damage, PINK1 engages with the mitochondrial import machinery. It is subsequently cleaved and degraded, but a damage-induced loss of mitochondrial import results in accumulation of PINK1 on the mitochondrial outer membrane where it can phosphorylate ubiquitin and activate Parkin-mediated mitophagy. Crystal structure (PDB 6EQI) analysis of PINK1 in complex with Ub (a T66V/L67N variant) revealed that Ub forms a bipartite interaction with the N lobe and the activation segment in the C lobe of the PINK kinase domain [25]. Tyr198 of the N lobe forms a hydrogen bond with Gly47 of Ub and hydrophobic interactions with Ile44 and Val70. The activation segment (residues 360–385) of the C lobe contacts the Ser65-containing loop of Ub, orienting Ser65 to a phospho-accepting position [25]. These interactions underlie the complex coordination between mitochondrial import and activation of mitophagy. Recently, the phosphatases PTEN-L (phosphatase and tensin homolog-long isoform) and PPEF2 (protein phosphatase with EF-hand domain 2) were found to antagonize the PINK1-dependent mitophagy by dephosphorylating pS65-Ub on the OMM [26–29]. Although pS65-Ub is routinely detected in yeast, there are no apparent structural homologs of PINK1 or PTEN-L/PPEF2 in yeast, and their functional analogs are yet to be identified.

S65 phosphorylation of Ub significantly impacts its structural and biophysical properties. Spectra generated by high-resolution 2D-NMR analysis reveal that pS65-Ub has two non-identical Ub conformations in dynamic equilibrium [30]. The major conformation comprises 70% of the population and resembles the unmodified Ub with notable resonance perturbations at the vicinity of Ser65. Crystallographic analysis of this significant species reveals that the phosphoryl group of pS65 forms a hydrogen bond with the backbone amide of Gln62, an interaction conserved in the unmodified Ub that links the hydroxyl group of Ser65 and Gln62. This major conformation of pS65-Ub exhibits a similar overall secondary structure compared to unmodified Ub, and the principal UBD recognition site, the hydrophobic patch including Ile44, is not grossly altered. In contrast, the remaining 30% of the population exhibits a significantly changed conformation compared to the unmodified Ub. This minor conformation exhibits a two-amino acid β 5-strand slippage downstream of the polypeptide chain, causing the C-terminal tail's contraction into the Ub core by two amino acids. The slippage stabilizes Leu73 and Arg74 residues and alters the interaction of the β 5 with β 1 and β 3 at the core. A new set of contacts are formed

between Ile44-Val70, Arg42-Arg72, Leu69-Phe4 and Leu71-Lys6, resulting in extension of the solvent-exposed loop that contains the pSer65, which could provide an opportunity for new surface interactions [30].

Given the drastic conformational perturbations associated with this minor conformation, it is not surprising that pS65-Ub exhibits some biochemical loss of function concerning specific conjugating enzymes. The Ser65-containing surface of Ub does not directly interact with E1 (activating) or E2 (conjugating) enzymes; and *in vitro* biochemical analysis did not reveal any loss of function with respect to thioester bond formation between pS65-Ub and E1 or E2 enzymes (13 of the 19 human E2 enzymes tested) [30, 31]. However, the discharge from E2 and Ub polymer assembly are altered in a subset of E2 enzymes. For example, the autoubiquitylation of human E2 enzymes UBE2R1(cdc34), UBE2E1(UbcH6) and UBE2T is significantly reduced with pS65-Ub. In addition, pS65-Ub impairs the production of unanchored K11-linked polyubiquitin by UBE2D1 [30]. When human E2 UBE2N was paired with the E2-fold proteins UBE2V1(UEVIA) or UBE2V2(Mms2), the assembly of pS65-Ub into free K63-linked chains was impaired [30]. Similarly, S65E phosphomimetic Ub impaired polymerization by yeast E2 enzymes Ubc1 and the Ubc13/Mms2 complex [16], with the notable caveat that phosphomimetic Ub is not a perfect proxy for pS65-Ub. Structural analysis of the yeast Ubc13/Mms2 complex shows that the phosphoryl group of pSer65 would clash with Glu18 of Mms2, thereby altering the orientation of Ub Lys63 at the active site of Mms2 [30]. In addition to its effects on E2 discharge, pS65-Ub can also impact specific E3 ligases. Ser65 phosphorylation inhibits conjugation reactions using the E2 enzymes UBE2D1 and UBE2D3 and the E3 ligase TRAF6, while the same E2 enzymes paired with the E3 ligase cIAP1 revealed no such defect compared to unmodified ubiquitin. In addition, Ser65 phosphorylation inhibits the formation of M1-linked (linear) polyUb chains by the E3 HOIP [30]. All of these biochemical analyses indicate that pS65-Ub can modify specific conjugation reactions in cells. Still, the regulatory impact remains unclear given the low abundance of unanchored pS65-Ub monomers relative to the pool of free ubiquitin. Future studies will need to address whether E3 ligases use pS65-Ub in conjugation reactions in cells.

Ser65 phosphorylation of ubiquitin is also reported to affect the recognition and activity of DUBs. One study measured the effect of Ser65 phosphorylation on DUB activities *in vitro* (31 human DUBs were tested) and found that the majority were strongly repressed in all di-Ub linkage types tested [31]. Similar observations were recorded using representative members from different DUB families and tested with phospho-tetra-Ub or high-MW phospho-polyUb as substrates [30]. Importantly, recent studies have reported that Ser65 phosphorylation inhibits the activity of USP30 [32, 33] – a mitochondria-localized DUB that antagonizes Parkin-mediated mitophagy. Surprisingly, Ser65 phosphorylation mildly enhanced some DUB activities, including OTULIN activity towards linear Ub polymers and USP4 and USP5 activity toward both Lys11 and Lys48 Ub polymers [31]. Ser65 phosphorylation also strongly enhanced the activity of USP16 on Lys33-linked polymers and OTUB1 and USP16 on Lys48linked polymers [31]. Taken together, these *in vitro* data indicate Ser65 phosphorylation of Ub has the potential to either repress or activate DUB activities, depending on the specific DUB and the linkage type of the polymer.

While several studies have reported biochemical data indicating how Ser65 phosphorylation of Ub alters conjugation and deconjugation reactions *in vitro*, less is known about its ability to modify recognition by UBDs and thus regulate the functional outcome of ubiquitin modification. One study used quantitative proteomics to compare interaction profiles of wildtype and S65E mono-ubiquitin in yeast [16]. This study found that S65E phosphomimetic Ub exhibits reduced interaction with specific UBD-containing proteins, such as the CUE-containing Cue5, UBA-containing Rup1, GAT-containing Tom1, and UIM-containing Vps27 [16]. In contrast, the UBA-containing Dsk2, Rad23 and Ede1 exhibit greater interaction with S65E Ub compared to WT Ub [16]. This analysis highlights the potential for Ser65 phosphorylation to modify UBD interactions and thus the effector outcomes of ubiquitylation.

Given all the biochemical data reported for how pS65-Ub affects conjugation, deconjugation, and ubiquitin-binding *in vitro*, there is strong potential for this modification to regulate various ubiquitin-dependent processes in a biological context. A recent study performed proteomic analysis of immunoprecipitated pS65-Ub and identified nuclear proteins involved in DNA repair, including DHX9, PRKDC XRCC6 and γH2A.X [29], suggesting that pS65-Ub may be associated with sites of DNA damage. Other pS65-Ub-associated proteins identified in this study include CSN1, MYCB2, PRS6/7 and PSA6, which are involved in the proteasome or autophagy pathways [29]. These findings emphasize the potential for Ser65 phosphorylation to regulate other ubiquitin-dependent processes. Still, additional functional and mechanistic analyses will be required to elucidate additional regulatory roles for this modification of ubiquitin. Some of the critical remaining questions that will fuel future research on pS65-Ub include:

- What pathways (besides mitophagy) are regulated by pS65-Ub?
- What kinases generate pS65-Ub in eukaryotes that lack PINK1 (e.g., yeast)?
- How does Ser65 phosphorylation change how the ubiquitin code is “read” by UBDs?

Ser57 phosphorylation of ubiquitin

pS57-Ub was the first phospho-Ub species discovered in a pioneering proteomic analysis of the ubiquitin-modified proteome of yeast [9]. Since then, other studies have reported pS57-Ub in yeast [34–36] as well as in different human cells and tissues – including breast cancer tissues [37], melanoma cells [38], and lung adenocarcinoma cells [39, 40]. However, alanine scanning mutagenesis of ubiquitin had revealed that Ser57 is not required for the essential functions of ubiquitin in yeast [17]. Indeed, a more recent deep mutational scanning analysis of ubiquitin revealed that the Ser57 position is fairly unique in that mutations at this position do not confer sensitivity across a panel of chemical stressors [41]. More directed studies have uncovered phenotypes associated with both S57A (phosphorylation resistant) and S57D (phosphomimetic) Ub variants. We found that S57D-Ub was associated with several stress-resistance phenotypes, conferring resistance to heat stress and toxic amino acid analogs such as canavanine and thialysine [35, 36]. Interestingly, S57D-Ub also conferred hypersensitivity to hydroxyurea, while expression of S57A-Ub conferred hypersensitivity to heat and oxidative stress [36]. Together, these findings underscore the

importance of the Ser57 position during conditions of stress. Interestingly, we also found that S57D-Ub exhibits a decreased half-life compared to WT ubiquitin. This finding is consistent with observations of reduced ubiquitin half-life in *ppz* phosphatase mutants, which exhibit elevated levels of pS57-Ub [35]. Following up on this observation, we found that S57D-Ub inhibits the activity of Doa4 [35] – a DUB that recycles ubiquitin before intraluminal vesicle formation in the ESCRT pathway. Based on these findings, we proposed that Ser57 phosphorylation regulates ubiquitin recycling efficiency in the endosomal sorting process.

In the absence of defined Ser57 ubiquitin kinases, some studies have analyzed pS57-Ub biochemical properties *in vitro* using purified recombinant pS57-Ub generated by biosynthetic incorporation of phosphoserine during translation in *E. coli*. One such study found that pS57-Ub is capable of hyper-activating Parkin *in vitro*, which is surprising given that pS65-Ub is a well-established Parkin activator in cells. This study raises the intriguing possibility that pS57-Ub could drive Parkin-mediated mitophagy independently of PINK1 and pS65-Ub [42], although this hypothesis is yet to be tested in a cellular context. Another study looked more broadly at how different phosphorylated Ub species affect conjugation cascades and DUB activities. This study found that Ser57 phosphorylation did not impact E2 charging nor the formation of linkage-specific polymers in conjugation assays using various E3 ubiquitin ligases [31]. However, when incorporated into dimers of different linkage types, pS57-Ub inhibited the activity of several DUBs – suggesting that Ser57 phosphorylation may regulate deconjugation, albeit in a way that is highly specific to the DUB and the linkage type of the polymer. (By comparison, pS65-Ub was a much more potent inhibitor of DUB activities *in vitro* [31].) Additional studies will be required to test if the biochemical effects of Ser57 phosphorylation reported *in vitro* correspond to the regulation of ubiquitin polymers in cells.

These biochemical studies make robust predictions about the regulatory consequences phosphorylation should have regarding ubiquitin conjugation and deconjugation, but how Ser57 phosphorylation may regulate interactions with UBDs remains unknown. NMR analysis of S57D-Ub did not reveal significant structural perturbations associated with this phosphomimetic substitution [35], suggesting that phosphorylation is unlikely to disrupt the structure of ubiquitin grossly. Most UBDs that have been characterized structurally interact with Ub on its hydrophobic patch (Leu8, Ile44, and Val70) [43]. However, relative to the hydrophobic patch, Ser57 is located on the opposite side of Ub (Figure 2), so phosphorylation at this position is unlikely to have a broad impact on Ub-UBD interactions. Interestingly, a few UBDs interact with the Asp58 patch on Ub, which includes Ser57 [44, 45]. For example, an A20-type zinc finger (A20-ZnF) domain from Rabex-5, a Rab5 GEF that regulates membrane fusion at recycling endosomes, interacts with the Asp58 patch of Ub [46, 47]. In these structures, Ser57 of Ub was observed to form polar interactions with Asn28 of Rabex-5. Likewise, a different A20-ZnF domain from ZNF216, a protein that regulates NF- κ B signaling, similarly binds to Ub at the Asp58 patch [45]. In both examples, the Asp58 patch engages in multiple polar interactions with the A20-ZnF domain. Importantly, UBDs that bind the Asp58 patch do not compete with interactions at the Ile44 hydrophobic patch, allowing for individual ubiquitin moieties to function as scaffolds in multi-protein complexes [48]. Together, these structural studies reveal the strong potential

for Ser57 phosphorylation to regulate interactions occurring at the Asp58 patch, which could have implications for how ubiquitin functions as a scaffold in the regulation of processes including endocytic trafficking and cell signaling.

A critical limitation to the investigation of pS57-Ub has been a general lack of understanding of the enzymes and physiological conditions that trigger its production in cells. Recently, we reported the identification of the first kinases capable of phosphorylating ubiquitin at the Ser57 position. Specifically, we screened for yeast kinases capable of phosphorylating ubiquitin in an *E. coli* co-expression system. This effort initially uncovered a single yeast kinase – the Snf1-related kinase Vhs1 – as a Ser57 ubiquitin kinase. However, subsequent rounds of screening uncovered additional yeast Snf1-related kinases capable of phosphorylating ubiquitin at the Ser57 position – including Sks1 (the Vhs1 paralog), Gin4, and Kcc4 [36]. Importantly, both Sks1 and Vhs1 were capable of generating pS57-Ub when expressed in yeast. Furthermore, phenotypes associated with Vhs1 or Sks1 overexpression were suppressed in the presence of S57A-Ub, while phenotypes observed in *sks1 vhs1* mutant cells were suppressed in the presence of S57D-Ub. These genetic interactions between Vhs1, Sks1 and the Ser57 position of ubiquitin indicate that the function of Sks1 and Vhs1 in yeast is related to pS57-Ub. Importantly, we also reported that human MARK and SIK kinases, members of the human family of Snf1-related kinases and homologous to Vhs1 and Sks1, exhibit Ser57 ubiquitin kinase activity *in vitro*. Another recent study performed large-scale *in vitro* screening to identify human ubiquitin kinases. This study found that monoubiquitin is generally recalcitrant to phosphorylation across a panel of human kinases [49], suggesting it may have evolved to be a poor kinase substrate by itself. However, several AGC kinases – including members of the PKA, PKC, PKG and RSK families – were capable of phosphorylating the Ser57 position of ubiquitin when GST was fused to both ubiquitin and kinase, providing a dimerization platform [49]. These results indicate that some AGC family kinases can phosphorylate ubiquitin when brought into proximity by binding to protein-ubiquitin conjugates. Interestingly, the substitution of amino acids flanking Ser57 (T55R and D58L) to create a PKA consensus recognition motif resulted in a ubiquitin variant that was a strong PKA substrate, independent of GST dimerization [49]. Thus, the suboptimal recognition motif is overcome only by recruitment via protein-protein interactions. Combined, these recent studies reveal a small number of Ser57 ubiquitin kinases – including MARK kinases which operate on ubiquitin polymers directly and AGC kinases that operate on ubiquitin when recruited to ubiquitin by other protein-protein interactions – but much additional work will be required to fully understand how these kinases function in a biological context.

PINK1-mediated production of pS65-Ub is tightly regulated and highly context-dependent, and there are indications that cellular production of pS57-Ub is likewise tightly regulated and context-dependent. First, deletion of many Ser57 ubiquitin kinases in yeast results in various stress-related phenotypes [36], suggesting they operate in the cellular response to different types of stress. For example, while both Sks1 and Vhs1 are required for the yeast response to oxidative stress, Vhs1 regulates sensitivity to canavanine and thialysine (toxic analogs of arginine and lysine, respectively). In contrast, Sks1 regulates sensitivity to hydroxyurea [36], illustrating that these kinases may have partially overlapping but also distinct cellular functions. Second, the *in vitro* activities of these kinases exhibit nuanced

differences in terms of specificity. For example, both Vhs1 and Sks1 showed activity toward mono-Ub but more robust activity toward polymers. Specifically, Vhs1 exhibited the greatest activity toward linear (M1-linked) and K29-linked ubiquitin polymers, while Sks1 exhibited the greatest activity toward linear (M1-linked) and K63-linked polymers [36]. Furthermore, MARK kinases exhibited activity toward linear ubiquitin tetramers, but not monomers, *in vitro*. Linkage specificity analysis of this activity revealed that MARK2 could phosphorylate linear, K11-, K63-, or K29-linked ubiquitin tetramers [36]. These linkage-type specificities may underlie unique regulatory functions and are consistent with the emerging picture that ubiquitin phosphorylation is tightly regulated and occurs in highly context-dependent manners in cells. Finally, the localization of these kinases is not well-characterized, and we hypothesize that stress-dependent subcellular localization may play an essential role in regulating localized production of pS57-Ub in cells. Ultimately, a combination of genetic analysis, biochemical characterization, and functional studies in cells will be required to define the specific contexts in which these kinases operate.

Currently, phosphatases that operate on pS57-Ub are still not defined. Our initial interest in pS57-Ub derived analysis of a phosphatase mutant – the *ppz* mutant of yeast, which lacks both Ppz1 and Ppz2. Yeast *ppz* mutants exhibit defects in the maintenance of ubiquitin levels (i.e., ubiquitin homeostasis) associated with increased levels of pS57-Ub [35]. While it is tempting to speculate that these function as Ser57 ubiquitin phosphatases in cells, we have been unable to express and purify recombinant Ppz complexes *in vitro* and thus unable to test if these Ppz phosphatases operate directly on phospho-ubiquitin. Therefore, further analysis will be required to define which phosphatases operate on pS57-Ub biochemically and in cells.

In recent years, progress has been made towards understanding the functional significance of pS57-Ub, but many important questions remain:

- What specific pathways are targeted by Ser57 ubiquitin kinases in cells?
- How do different kinases operate on different ubiquitin forms – including monomeric, unanchored polymeric, and protein-conjugated ubiquitin?
- What subcellular and stress-dependent contexts are associated with pS57-Ub production?
- What phosphatases operate on pS57-Ub?
- How does Ser57 phosphorylation change how the ubiquitin code is “read” by UBDs?

pT12-Ub and other phosphorylated ubiquitin species

Compared to pS65-Ub and pS57-Ub, significantly less is known about other phosphorylated ubiquitin species. Many additional phosphorylation sites on ubiquitin have been detected in proteomic studies, including Thr7, Thr12, Thr14, Ser20, Thr22, Tyr59 and Ser66 (summarized in [50] and [51]). *In vitro* studies revealed that pS20-Ub converts UBE3C from a dual-specificity E3 ubiquitin ligase to a K48-specific ligase [31]. pS20-Ub was also reported to inhibit the activity of some DUBs, including cleavage of linear (M1-linked)

di-Ub by CYLD, cleavage of K33-linked di-Ub by USP6 and USP25, and cleavage of K63-linked di-Ub by TRABID [31]. Additionally, pS20-Ub broadly inhibited cleavage of K48-linked di-Ub across a panel of DUBs [31]. This biochemical analysis underscores the regulatory potential of ubiquitin phosphorylation at Ser20, although the biological significance of pS20-Ub has not been determined.

Ubiquitin phosphorylated at Thr12 (pT12-Ub) was first detected in proteomic experiments comparing tissue samples from hepatocellular carcinoma and healthy patients [52]. Subsequent studies identified pT12-Ub in several different biological contexts, including: (i) proteomic analysis of nuclear proteins from Epstein-Barr virus-transformed B-lymphocyte cells [53], (ii) phosphoproteomic analysis of HeLa and K562 cell lines [54], and (iii) phosphoproteomic analysis of mitotically arrested HeLa cells [55]. Structurally, Thr12, along with Lys6, Lys11, Thr14, and Glu34 all contribute to a surface feature known as the TEK box [3], which is proposed to play a role in orienting ubiquitin for the formation of K11 linkages. For example, the TEK box is critical for forming K11-linked ubiquitin polymers by APC [57]. Thus, phosphorylation at Thr12 has the potential to regulate the formation of K11 linkages by APC and, by extension, the cell cycle. Another potential function of pT12-Ub involves the activity of HOIL-1, one of the subunits of the LUBAC complex. HOIL-1 was recently reported to catalyze the formation of oxyester bonds between ubiquitin and Ser or Thr residues on substrates [58]. Interestingly, HOIL-1 generated ubiquitin dimers in vitro that were conjugated at the Thr12 position [58], raising the interesting possibility that pThr12-Ub could antagonize ester-linked polymerization of Ub by atypical E3 ligases. By extension, phosphorylation at other positions could likewise regulate ester-linked polymerization of ubiquitin at other Ser and Thr residues. The development of new tools and reagents to detect pT12-Ub will be needed to determine if this type of regulation occurs in cells.

Recently, pT12-Ub has garnered attention for its role in regulating the DNA damage response [56]. DNA damage triggers ubiquitin modification of histone H2A at Lys13 and Lys15, via the RNF168 ubiquitin ligase. Ubiquitin modification of H2A at Lys15 is recognized by 53BP1, a factor that promotes non-homologous end-joining. Interestingly, DNA damage-induced production of pT12-Ub was reported to block 53BP1 recruitment and promote homologous recombination. Other DNA repair factors, such as RAD51, were still able to bind in the presence of pT12-Ub. Structural analysis revealed that phosphorylation at Thr12 disrupts the Ub-53BP1 interface [56]. To date, specific kinases and phosphatases that operate on Thr12 have not been reported.

Phosphorylation of Ubiquitin-like Modifiers

Ubiquitin-like proteins (UBLs) play essential roles in cellular function [59]. Humans encode 9 UBL families: ATG8, ATG12, FAT10, FUBI, ISG15, NEDD8, SUMO, UFM1, URM1 [60] (Figure 3A). Sequence alignment analysis reveals limited conservation at sites corresponding to Ser57 and Ser65 ubiquitin (Figure 3B–C). For example, sequence analysis of yeast UBLs revealed that the site corresponding to Ser57 in yeast was often either a potential phosphorylation site (Ser, Thr) or a phosphomimetic residue (Glu) (Figure 3B). This trend was not observed in human UBLs (Figure 3C), although it is noteworthy that the

human UBLs contained an Ala residue at the site corresponding to Ser57. Interestingly, yeast and human SUMO homologs all contain phosphomimetic Asp at the position corresponding to Ser65 in ubiquitin. Overall, this analysis suggests that the potential for phospho-regulation of UBLs at sites corresponding to Ser57 and Ser65 of Ub is limited.

For most human UBLs phosphorylated species have been detected in proteomic studies (Table 2). ATG8 phosphorylation sites were partially characterized, and several kinases for ATG8 family members are known. PKA phosphorylates Ser12 on LC3A, and the phosphomimetic S12D variants reduced LC3A recruitment to autophagosomes, while S12A substitutions increased recruitment to autophagosomes [61]. LC3B and LC3B2 are phosphorylated on T6, T29 and T50. PKC phosphorylates T6 and T29, but the phosphorylation of these sites does not affect autophagy. In contrast, STK3, STK4 [62], NEK9 and PKC ζ [63] phosphorylate T50 to induce autophagy [62]. TBK1, a kinase known for its significant role in autophagy [64–66] and innate immune signaling [67, 68], can phosphorylate LC3C and GABARAPL2 (LC3C at S93 and S96; GABARAPL2 at S87 and S88) [69]. LC3C and GABARAPL2 phosphorylation ensure the steady formation of autophagosomes. Phosphorylation of LC3C and GABARAPL2 blocks their interaction with ATG4 and subsequently prevents the removal of LC3C and GABARAPL2 from premature autophagosomes [69]. Impaired autophagy has been linked to several diseases, including neurodegeneration, inflammatory disorders and cancer [70]. Therefore, uncontrolled phosphorylation of ATG8 family members might be crucial to understand the molecular pathways resulting in these pathological phenotypes.

Compared to ATG8 family members, much less is known about phosphorylation of protein-conjugated UBLs. Due to the high number of proteomics datasets available, phosphorylation sites are known for most of these UBLs (Table 1). However, the function and regulation of these phosphorylation events remain unknown. The SUMO family is the best-studied UBL family, and the Mann lab and colleagues described the first SUMO phosphorylation site in 2008. They observed conserved phosphorylation of SUMO at Ser2 in humans and lower eukaryotes such as drosophila and yeast [71]. SUMO phosphorylation at Ser2 is of particular interest since this residue is not conserved amongst all SUMO family members. Ser2 is present in SUMO1, SUMO3 and SUMO5 but not in SUMO2 or SUMO4. Ser2 SUMO phosphorylation might play a role in differentiating SUMO2 and SUMO3, which differ by only three amino acids. SUMO5 is highly expressed in testes and peripheral blood leukocytes but is missing in many other human tissues and cell lines [72]. This highly restricted expression might be the reason why SUMO5 phosphorylation has not been described so far.

The regulatory significance of UBL phosphorylation remains understudied, and deciphering its significance will require new research. Most proteomics datasets revealed UBL phosphorylation under steady-state conditions. Thereby, datasets inherently lack information about context-specific UBL phosphorylation dynamics. ISG15 expression, for instance, is induced by interferon stimulation [73], making it a potential blind spot of phospho-proteomic analysis. One of the significant tasks of future research will be to identify the upstream signaling events resulting in UBL phosphorylation. The cell-type and stimuli-specific expression of UBLs likely results in an incomplete picture of the UBL

phosphorylation landscape. The majority of UBL phosphorylation sites were identified in laboratory cell lines, which only approximate cellular behavior *in vivo*. The origin of the cell lines and their signaling context should be considered when studying UBL phosphorylation. In addition to the upstream signaling events, it will be of equal interest to elucidate UBL phosphorylation's biological function. Phosphomimetic UBL variants might shed some light on their biological function, but these mutations cannot reflect the spatiotemporal changes of UBL phosphorylation. In this context, identifying the UBL-targeting kinases and phosphatases will be the key to understand the biochemical and physiological functions of UBL phosphorylation.

Concluding Remarks and Remaining Questions

Despite decades of intense research on the ubiquitin code, the significance of Ub phosphorylation has only recently come into focus. So why has it taken so long to appreciate this feature of the ubiquitin code? One reason is the low abundance of phosphorylated ubiquitin species in cells. Given the abundance of ubiquitin, its essential regulatory functions in eukaryotic cells, and the potential for phosphorylation to modify these functions, it is tempting to speculate that ubiquitin may have evolved to avoid recognition by kinases to maintain phosphorylated species at low stoichiometry. Consistent with this speculation, kinase activity toward ubiquitin appears to be tightly regulated and highly localized. For example, PINK1 exhibits very low activity and is degraded until mitochondrial damage triggers its accumulation and activation on the mitochondrial outer membrane, leading to localized production of pS65-Ub in the proximity of aberrant import complexes. Such deliberate, context-dependent production of phosphorylated ubiquitin underscores its regulatory potency as well as the challenges associated with dissecting its functions.

One factor limiting progress in this field is a lack of reagents for detection of pUb species. Although many pUb species can be detected by mass spectrometry, commercial antibodies are currently only available for the detection of pS65-Ub. These antibodies have been critical for advancing the understanding of the PINK1-Parkin pathway and the role pS65-Ub plays in mitophagy. Unfortunately, similar antibodies are not commercially available for other pUb species. Although antibodies detecting pS57-Ub have been described and validated they lack the sensitivity for detection in biological samples [35, 36]. Development of sensitive, commercially-available antibodies for recognition of a variety of pUb species will be an important step forward that will catalyze new mechanistic insights at this important frontier.

Despite these limitations, several themes are emerging that could help to guide ongoing and future research in this area. One emerging theme is that ubiquitin phosphorylation appears to be involved in stress responses: pS65-Ub regulates mitophagy in response to mitochondrial damage, pS57-Ub regulates the oxidative stress response, and pT12-Ub regulates the DNA damage response. All of these stress responses were previously known to be Ub-dependent, but the phosphorylation of ubiquitin in response to specific stressors suggests its function is being modified to optimize the cellular response to various insults and environmental challenges. It is tempting to speculate that ubiquitin phosphorylation may provide a mechanism for rapid activation of stress responses that must be tightly regulated and kept "off" in normal circumstances but must also be switched on quickly to restrict

cellular damage in conditions of stress. This is definitely the case for pS65-Ub in mitophagy [13], but additional work will be required to determine if ubiquitin phosphorylation at other positions likewise facilitates feedforward activation during the response to cellular stress.

Generally, an improved understanding of how and where pUb species are produced in cells will require identifying kinases and phosphatases that regulate production in response to stress. Substantial progress will likely require the development of new tools and reagents for the detection and quantification of pUb species. Although several studies have revealed biochemical insights into how Ub phosphorylation can influence conjugation and deconjugation reactions *in vitro*, much work is still needed to pinpoint which reactions are regulated in a physiological context. Furthermore, it remains unclear to what extent phosphorylation regulates Ub recognition. In this review, we have used structural information to speculate how specific phosphorylation events on Ub might interfere with known ubiquitin binding domains. Additionally, some ubiquitin binding domains may specifically recognize pUb species, and the discovery of such domains could help elucidate how phosphorylation regulates Ub biology. These challenges and unknowns provide a fertile frontier ready for exciting discoveries that have potential to transform our understanding of the ubiquitin code and its role in proteome remodeling and the cellular stress response.

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Abbreviations

APC	anaphase promoting complex
AQUA	absolute quantification mass spectrometry
DUB	deubiquitylase
ESCRT	endosomal sorting complexes required for transport
LUBAC	linear ubiquitin assembly complex
OMM	outer mitochondrial membrane
MARK	microtubule affinity regulating kinase
PINK1	PTEN induced putative kinase 1
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
pS65-Ub	Ser65-phosphorylated ubiquitin

pS57-Ub	Ser57-phosphorylated ubiquitin
pT12-Ub	Thr12-phosphorylated ubiquitin
PTM	post-translational modification
SUMO	small ubiquitin-like modifier
Ub	ubiquitin
UBD	ubiquitin binding domain
UBL	ubiquitin-like modifier
WT	wildtype

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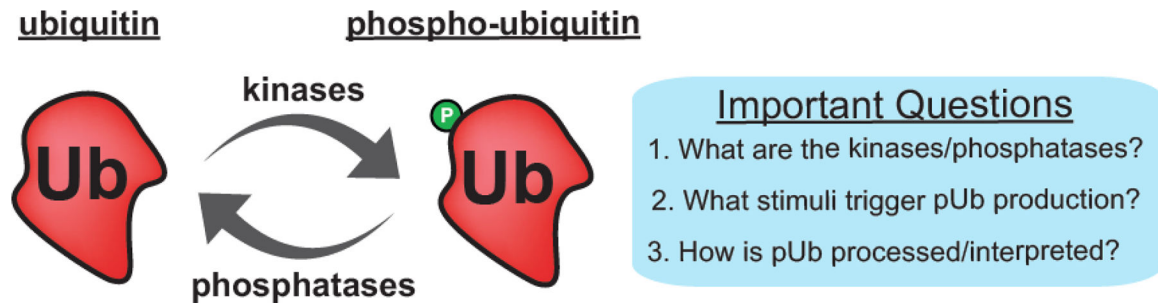
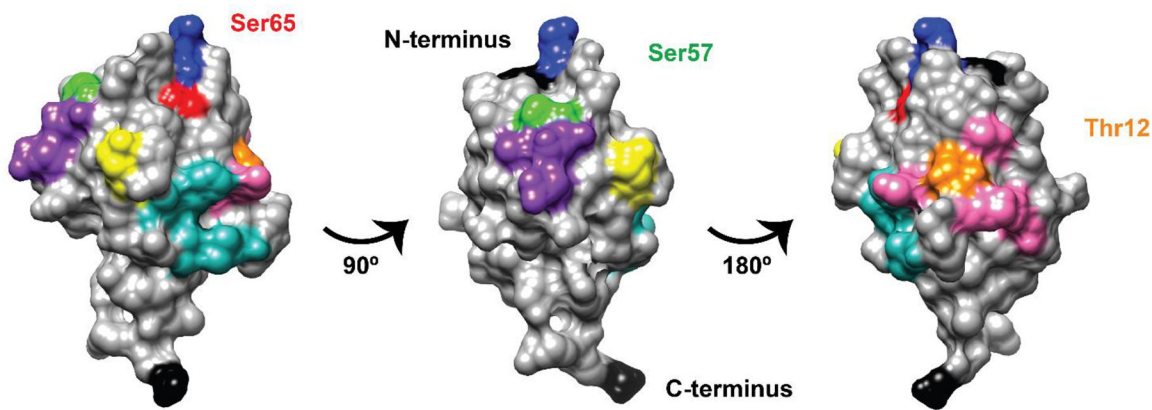


Figure 1. Phosphorylation has the potential to regulate many aspects of ubiquitin function. The blue box highlights several important questions that remain to be answered in this new arena of regulatory biology.



<u>Phosphorylation Sites on Ubiquitin</u>			
Ser65	Ser57	Thr12	
(left panel)	(middle panel)	(right panel)	
<u>Reactive Features of Ubiquitin</u>			
Lys48	Lys63	N-terminus	C-terminus
<u>Ubiquitin Surface Features</u>			
<u>Ile44 Patch</u>	<u>Asp58 Patch</u>	<u>TEK Box</u>	
Leu8	Arg54	Lys6	
Ile44	Thr55	Lys11	
His68	Ser57	Thr12	
Val70	Asp58	Thr14	
		Glu34	

Figure 2. Analysis of phosphorylation sites relative to other functional surfaces on ubiquitin. The structure of ubiquitin (PDB ID: 1UBQ) was analyzed using UCSF Chimera (ver. 1.15), positioning the molecule around a vertical N-to-C terminal axis. (N- and C-termini are colored black.) Sites of phosphorylation are colored as follows: Ser65 = red, Ser57 = green, Thr12 = orange. Functional surface features of ubiquitin are colored as follows: K48 = yellow, K63 = dark blue, Ile44 hydrophobic patch residues = teal, Asp58 patch residues = purple, TEK box residues = pink. The molecule is rotated to provide views focused on Ser65 (left), Ser57 (middle) and Thr12 (right).

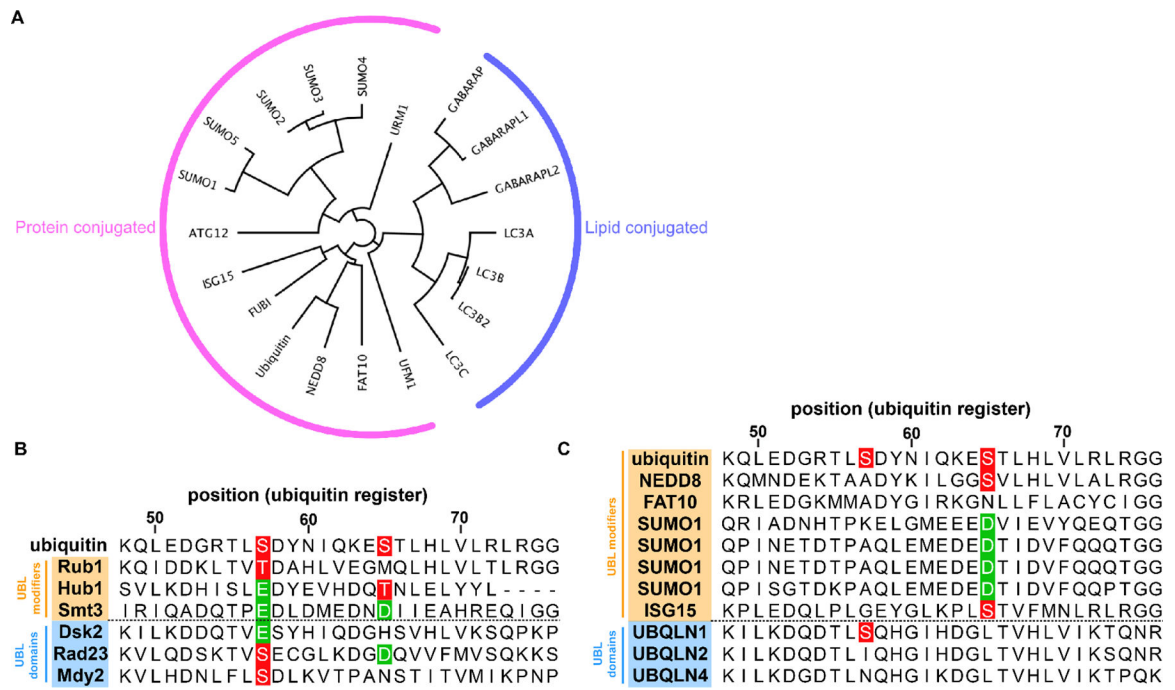


Figure 3. Analyzing the potential for phosphoregulation of UBLs. (A) Phylogenetic relationship of ubiquitin and human UBLs. Branch lengths are proportional to phylogenetic distances. (B and C) Yeast (B) or human (C) amino acid sequences for ubiquitin (top row), ubiquitin-like (UBL) modifiers (shaded orange) or UBL domains in larger proteins (shaded blue) were aligned using Clustal Omega. The alignment was visualized using Jalview, a portion of which (corresponding to the indicated register for ubiquitin) is shown. Highlighted are the positions corresponding to Ser57 and Ser65 in ubiquitin. Positions shaded red indicate a residue that can be phosphorylated (Ser or Thr) while positions shaded green are phosphomimetic (Glu or Asp). For several yeast UBLs (Rub1, Rad23 and Mdy2) the position corresponding to Ser57 is a potential site of phosphorylation, while for others (Hub1, Smt3 and Dsk2) this residue is a phosphomimic (glutamate).

Table 1.

Summary of phosphorylation sites on ubiquitin. Phosphorylation of human ubiquitin is represented in the top rows of the table (shaded green) while yeast ubiquitin is represented in the bottom rows of the table (shaded orange). This table represents phosphorylation events that are the best characterized (to date) and have regulatory significance with regards to ubiquitin function. Other phosphorylation sites on ubiquitin have been detected, but the significance of other events remains to be elucidated.

	Site	Biological function	Kinases	References
Ubiquitin (human)	Ser65	Regulation of mitophagy	PINK1	[11, 12, 74]
	Ser57	Unknown	MARK1–4, SIK1–2 PKA, PKC, PKG	[36]
	Thr12	DNA damage response	unknown	[56]
Ubiquitin (yeast)	Ser65	unknown	unknown	
	Ser57	stress responses ubiquitin homeostasis	Vhs1, Sks1 Gin4, Kcc4	[35, 36]
	Thr12	unknown	unknown	

Table 2:

Phosphorylation sites of UBLs according to PhosphoSitePlus [75, 76]. UBLs, except for FUBI and ATG8, are reversibly conjugated to lysine residues on target proteins via their C-terminal glycine motifs. FUBI is classified as a protein conjugating UBL, but no FUBI substrates are known to date [77]. ATG8 family members are not conjugated to proteins but the lipid phosphatidylethanolamine (PE) to induce autophagy [78, 79].

Family	Proteins	Phosphorylation sites	Substrate
ATG8	LC3A	S12; S92	lipid (PE)
	LC3B	T6; T29; T50	
	LC3B2	T6; T29; T50	
	LC3C	S93; S96	
	GABARAP	Y49; Y61	
	GABARAPL1	Y5; Y49; Y61	
GABARAPL2	S10; S39; S87; S88		
ATG12	Atg12	S41; T65; T70	protein (lysine)
FAT10	FAT10	S64; T73; T77; S109; S110; S111; Y148; Y161	protein (lysine)
FUBI	FUBI	---	unknown
ISG15	ISG15	S22; S26; S50	protein (lysine)
NEDD8	NEDD8	T7; T9; T20; Y45; T55; Y59; S65	protein (lysine)
SUMO	SUMO1	S2; S9; S10; S32; T76	protein (lysine)
	SUMO2	T12; S28; T38; S54	
	SUMO3	S2; T12; S27; T37; S53	
	SUMO4	S28	
	SUMO5	---	
UFM1	UFM1	Y18; S72	protein (lysine)
URM1	URM1	---	protein (lysine)